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ELK and LERK-2 in developing kidney and microvascular endothelial assembly

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ELK and LERK-2 in developing kidney and microvascular endothelial assembly. Eph family receptor tyrosine kinases direct neuronal cell targeting, bundling and intercellular aggregation activity, yet their role in mammalian kidney development has been unexplored to date. We recently identifed expression of ELK (Eph-like kinase) receptors in cultured human renal microvascular endothelial cells (HRMEC), and showed that ELK mediates their *in vitro* assembly into capillary-like structures in response to the exogenous ligand, LERK-2. Here we identify expression of the ELK ligand, LERK-2, in HRMEC and in primitive vascular structures of developing murine kidney. ELK and LERK-2 are expressed on endothelial progenitor cells of primitive microvasculature in a pattern similar to that of the VEGF receptor, flk-1. ELK, LERK-2 and flk-1 antigens are also displayed on the branching ureteric bud epithelium; ELK and LERK-2 expression persists in mature collecting ducts, glomeruli and arterioles. To explore whether renal-derived endothelial cells may distinguish LERK-2 from the angiogenic Eck ligand, LERK-1 (B61), and whether endothelial cells from different sources may distinguish among Eph receptor ligands, we compared HRMEC and human umbilical vein endothelial cell (HUVEC) responses in an *in vitro* capillary-like assembly assay. HRMEC endothelial cells assembled capillary-like structures in response to LERK-2, but not LERK-1, under conditions that promoted HUVEC to assemble in response to LERK-1, but not LERK-2. Therefore, responses mediated through specific Eph family receptors (ELK and Eck) are discriminated by endothelial cells from different vascular bed sources. ELK and its ligand, LERK-2, are spatially and temporally coordinated in expression and may function in morphogenesis of the renal microvasculature.

The highly specialized microvascular circulation of the mammalian kidney develops through integrated processes of cell migration, aggregation, morphogenesis and differentiation [1]. Vascular progenitor cells assemble into an architecturally-defined relationship with the epithelial structures that develop concurrently, in both glomerular and peritubular microvasculature [2, 3]. The molecular controls regulating these processes remain poorly defined, particularly those that establish microvascular organization in apposition with epithelium. Yet this intimate interrelationship is required for the functional coupling of filtration, reabsorption, and countercurrent processes.

Early data from chimeric transplantation experiments lead to the conclusion that the renal microvasculature develops by angiogenic budding, branching and migration of vascular endothelial cells from extrarenal sources [4]. Recent evidence indicates that the endothelial progenitor cells that contribute to glomerular microvasculature are dispersed in the metanephric mesenchymal tissue [1]. These cells are marked by their expression of the flk-1 receptor for vascular endothelial growth factor (VEGF) [5]. In response to yet undefined signals, these flk-1 expressing cells assemble into glomerular capillaries through a vasculogenic process. Based on these observations, it appears that very early steps in renal endothelial differentiation are marked by flk-1 expression, before these flk-1 expressing cells are incorporated into identifiable vascular structures.

Other studies have implicated VEGF and its receptors in renal vascular development. Messenger RNA for VEGF was localized to glomerular epithelial cells, while mRNA for its receptor, flk-1 (KDR), was restricted to glomerular endothelial cells; mRNA for a different VEGF receptor, flt-1, was identified in both glomerular and peritubular endothelium [6].

Recent evidence has suggested critical roles for other receptor tyrosine kinases in vascularization of mammalian kidney [7]. PDGFβ receptor mutant mice show failure of glomerular development [8]. Landels et al described *tie-2* expression in E11 metanephros, presumably in endothelial lineage cells [9]. Animals nullizygous for expression of the endothelial orphan receptor kinases, *tie-1* and *tek (tie-2)*, do not survive vascularization failure sufficiently long enough to evaluate their roles in renal microvascular development, but these kinases are critical for endothelial development and integrity at other sites [10, 11].

A distinct class of receptor tyrosine kinases of the Eph family have recently been assigned important roles in regulating cell aggregation, targeting and differentiation responses, particularly in the central nervous system where their functions have been most extensively explored [12, 13]. Eph family receptors include at least 13 distinct proteins expressed in species from *Xenopus laeveas* to *Homo sapiens* in highly tissue-restricted distributions during development [14]. A subclass of these receptors has been implicated in directing the retinotectal projections from developing neurons, through responses to geographically defined gradients of the receptors and their ligands [15–17]. These receptors mediate nonproliferative signals and have been localized to processes of migrating axons, concentrated at sites and during stages of development of intercellular functional contacts [18].

To date, seven different membrane associated ligands for Eph

Table 1. Ligands and their interactions with Eph family receptors

Ligands	Human	Mouse	Rat	Chicken	Quail	Frog	Fish
	Receptors						
LERK-1 (B61)	ECK						
LERK-2 (EFL-3, mCEK5-L, Elk-L)	ELK (NET)	mElk	Elk	Cek6		Xek	
	HEK2	Sek4 (Mdk5)	Tyro6	Cek10			
	ERK (HEK5)	Nuk (Sek3)	Tyro5	Cek5	QEK5	Xelk	Rtk3
LERK-3 (Ehk-1, EFL-2)	ECK	Myk (Sek2)					Rtk2
LERK-4	HEK	Mek4	Tyro4	Cek4		Pagliaccio	
LERK-5 (Htk-L, mELF-2)	ELK (NET)	mElk	Elk	Cek6		Xek	Rtk3
	HTK	Myk1	Tyro11				
	(HEK13)	Sek4 (Mdk5)	Tyro6	Cek10			
	HEK2						
LERK-6 (ELF-I, Cek7-L)	HEK	Mek4	Tyro4	Cek4		Pagliaccio	
	HEK7	Bsk	Ehk1	Cek7			
	HEK8	Sek	Tyro1	Cek8			Rtk1
LERK-7 (AL-1)	HEK7	Bsk	Ehk1	Cek7			
?	EPH						
?	HEK9			Cek9			
?	EEK		Eck				
?	HEK1	Mdk1	Ehk3 (Ebk)				
?			Ehk2				

Interspecies orthologues are indicated by their position on the same line.

related kinases (LERKs) have been characterized, sharing structural features within the extracellular domain [19]. These proteins are functionally subdivided into two groups that have overlapping affinities for several members of the receptor families, as is summarized in Table 1. One group, including LERKs 2 and 5, include transmembrane domains and highly conserved intracellular domains without a known enzymatic function or recognized signaling domain motif. The second group, including the prototype family member, B61 (LERK-1), are membrane associated through glycosylphosphatidylinositol linkages. These ligands are highly specific in their tissue distribution during development. The AL1/RAGS (LERK-7) protein is expressed in a gradient distribution that has been implicated in targeting receptor-bearing retinal neurons to appropriate projections on the tectum [15, 17, 20].

Recent evidence has implicated the Eph family receptor, Eck, in angiogenic responses in vascular endothelial cells. The ligand for Eck is B61 (LERK-1), a TNFα-induced product of endothelial cells that appears to mediate angiogenic responses to TNFα in a rabbit corneal angiogenesis assay through its interaction with the Eck receptor [21]. We have recently identified expression of a second endothelial Eph family receptor, ELK, in human renal microvascular endothelial cells (HRMEC) (Stein et al, manuscript submitted). Alternatively spliced isoforms are expressed in HRMEC and in developing murine embryos, and ELK receptors mediate assembly of HRMEC into capillary-like structures in response to the ELK ligand, LERK-2.

Given the roles Eph family receptors have in directing developing neural cell organization, coupled with our recent recognition of ELK receptor expression in HRMEC, we explored the distribution of ELK receptors and their ligand, LERK-2, in developing and adult mammalian (murine) kidney. Finally, we have addressed whether distinct Eph receptor ligands may evoke different responses from endothelial cells derived from the renal microcirculation, compared with nonrenal endothelial cells.

Methods

Northern blot analysis

Total RNA was prepared from cultured cell monolayers by the guanidine isothiocyanate-CsCl gradient method [22]. A 550 bp extracellular domain *ELK* fragment (*Eco*RI/*Bgl*II), a full length LERK-2 cDNA insert [23], or a human G3PDH (constitutively expressed control) cDNA fragment were labeled by the random primer method (Prime-it; Strategene, San Diego, CA, USA) and Northern blots were hybridized with 1.5×10^6 cpm ml^{-1} for 16 to 20 hours at 42°C. After hybridization, blots were washed with $0.2 \times$ SSC/0.1% SDS at 65°C, or as indicated, and exposed to Kodak XAR film (Kodak, Rochester, NY, USA) using intensifying screens for the periods indicated in the Figure legends.

LERK-2 and ELK antibodies/LERK-2/Fc and LERK-1/Fc fusion proteins

Rabbit anti-LERK-2 antibodies were generated using an 18 amino acid peptide representing sequences in the extracellular juxtamembrane spacer domain that is unique to LERK-2 among Eph family ligands (amino acids 244-261), as described [23]. Rabbit anti-ELK antibodies were raised against a recombinant antigen comprised of the entire extracellular domain of rat ELK fused with human IgG_1 Fc domain sequences, as described [23].

Transient transfections and immunoprecipitation of expressed ELK receptors

COS 7 cells were passaged in DME growth media containing 10% defined supplemented calf serum (HyClone Laboratories,

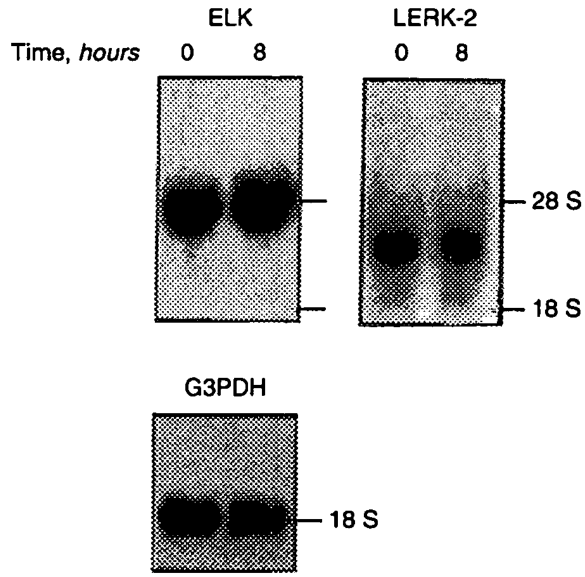


Fig. 1. *Human renal microvascular endothelial cells express ELK and LERK-2.* HRMEC were exposed to phorbol myristate acetate (PMA, 20 ng ml^{-1}) for zero or eight hours, as indicated. Total RNA (30 μg) was separated and probed on Northern blots, as described in the **Methods** section, using radiolabeled probes for human ELK, human LERK-2 or G3PDH, as indicated. Blots were exposed to Kodak XAR film for ten hours, using enhancing screens.

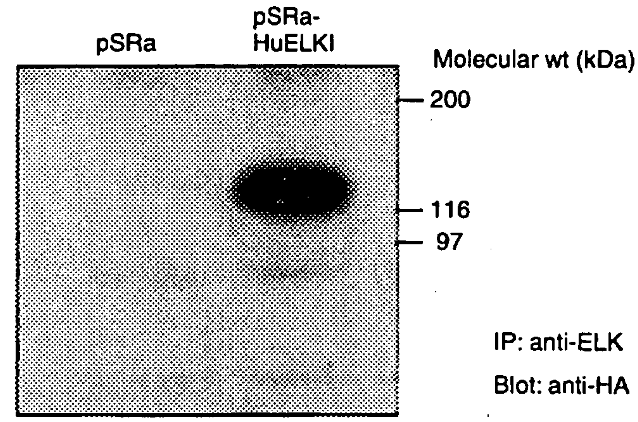


Fig. 2. *ELK antibodies immunoprecipitate endothelial ELK receptors.* COS 7 cells were transfected with either control plasmid (pSRα) or an expression plasmid encoding HA-tagged human ELK receptors (pSRα-HuELKI), lysed and lysates immunoprecipitated with anti-ELK antibodies. Immunoprecipitates were separated on 8% SDS polyacrylamide gels, transfered to Western blots and probed with 12CA5 (Boehringer) antibody against the HA epitope.

Logan UT, USA). Cells were transfected with an expression plasmid pSRα-HuELKIHA [that drives high level expression of the human ELK receptor modified by addition of a C terminal hemagglutinin (HA) epitope tag], using a calcium phosphate-DNA precipitation method [24]. After 54 hours, cells were placed on ice, washed twice with ice cold PBS/1 mM sodium ortho-vanadate/1 mM PMSF and immediately lysed in 1 ml buffer AB (20 mM TrisCl, pH 7.5/50 mM NaCl/0.2% Triton X-100/0.25% Na deoxycholate/0.5% SDS/5 mM NaF/1 mM EDTA/1 mM ortho-vanadate/1 μg ml^{-1} leupeptin/2 μg ml^{-1} aprotinin/1 mM PMSF). Lysates were clarified by centrifugation, and ELK receptors were immunoprecipitated using the rabbit anti-ELK antibodies. Cleared cell lysates were incubated overnight at 4°C with rabbit anti-ELK recognizing the extracellular domain of rat ELK [23]. Immune complexes were recovered by batch adsorption to Protein A-sepharose for 60 minutes at 4°C. Sepharose beads were washed by serial suspension in buffers and collected by centrifugation: two washes with RIPA-buffer (BSA, 1 mg ml^{-1}/10 mM TrisCl, pH 7.5/150 mM NaCl/5 mM NaF/1% DOC/1% Triton X-100/0.1% SDS/1 μg ml^{-1} leupeptin/2 μg ml^{-1} aprotinin/1 mM PMSF] and one subsequent wash with RIPA buffer omitting BSA.

Precipitated immune complexes were resolved by SDS-PAGE, transferred to Immobilon-P transfer membranes (Millipore), and blocked in 5% non-fat dry milk in Tris-buffered saline, (20 mM Tris, pH 7.6/137 mM NaCl/0.1% Tween-20) for one hour at room temperature. Blots were incubated with the murine monoclonal antibody 12CA5 (Boehringer), followed by incubation with horseradish peroxidase-conjugated goat anti-mouse IgG antibodies (Boehringer). Membranes were washed, then developed using a chemiluminescent reaction (ECL; Amersham Corp.) according to the manufacturer's protocol.

Endothelial assembly into capillary-like structures

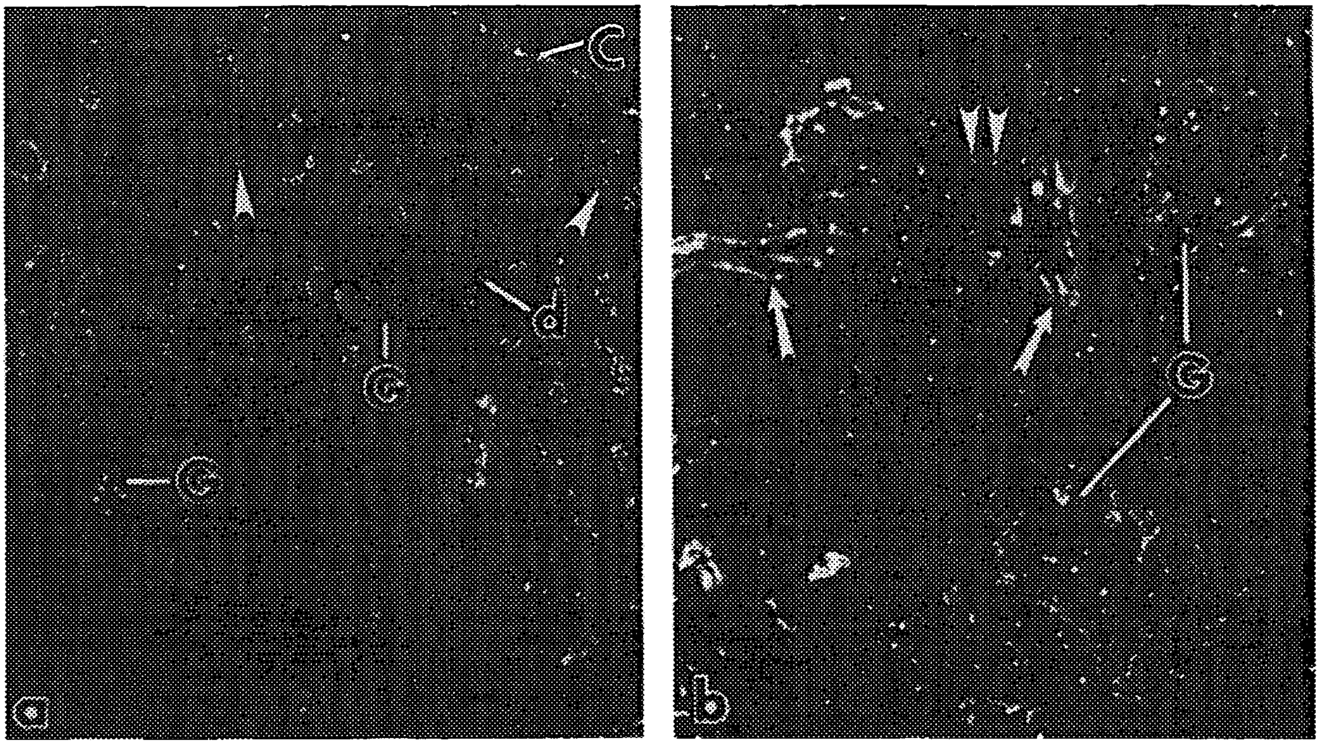
Twelve well plates (Falcon) were coated with thin layers of Matrigel, as described (Collaborative Biomedical Products; Becton Dickinson, Bedford, MA, USA). HRMEC or HUVEC were plated at a density of 4 × 10^4 cells/well in DME containing 1% fetal bovine serum. At the time of plating, agonists or control peptides were added at the indicated concentrations in the absence or presence of clustering antibodies, as above. Cells were incubated at 37°C for times indicated and photographed under phase microscopy (Diaphot-TMD, Nikon) using TMX-100 film (Kodak).

Immunofluorescence

Fresh, unfixed kidney samples from newborn and adult mice were snap-frozen in 2-methylbutane cooled in a dry ice-acetone bath. Cryostat sections (5 μm thick) were air-dried, fixed with 100% methanol at −20°C for 10 minutes, washed three times with PBS, and then blocked with 10% normal goat serum for 10 minutes. Slides were then labeled with rabbit anti-ELK, anti-LERK-2 or anti-flk-1 IgG [Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA; this antibody (designated C-1158) is an affinity-purified rabbit polyclonal IgG raised against a GST fusion protein containing the C-terminal amino acids 1158-1345 of mouse flk-1]. Sections were again washed with PBS, and then incubated with goat anti-rabbit IgG-rhodamine (Cappel-Organon Teknika Corp., Durham, NC, USA). Slides were examined by epifluorescence in a Leitz Aristoplan photomicroscope (Leica, Inc., Deerfield, IL, USA).

WGA lectin recovery of ELK and Eck receptors/phosphotyrosine Western analysis

Following exposure to ligands as indicated in the Figure legends, cells were lysed in buffer WG (50 mM HEPES pH 7.5/50 mM NaCl/5 mM EDTA/1% Triton X-100/1 mg ml^{-1} BSA/1 mM ortho-vanadate/1 μg ml^{-1} leupeptin/2 μg ml^{-1} aprotinin). Insoluble material was cleared by centrifugation (13,000 × g, 4°C, 10 min) and cell lysates were incubated with agarose-bound triticum



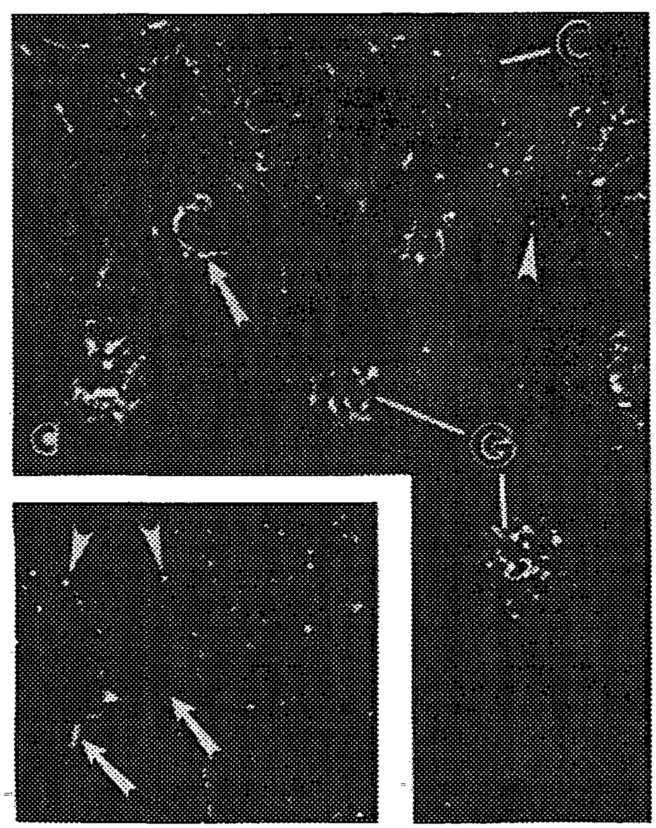


Fig. 3. *Immunolocalization of ELK, LERK-2 and flk-1 in newborn murine kidney.* Cryostat sections of newborn mouse kidney sequentially labeled with rabbit anti-ELK (**A**), anti-LERK-2 (**B**), and anti-flk-1 (**C**) IgGs, and goat anti-rabbit IgG-rhodamine, as described in the **Methods** section. (A) Anti-ELK binds intensely to foci on cell surfaces in mesenchymal areas (arrowheads), and weakly to maturing glomeruli (G). There is also some binding to apical regions of collecting duct epithelial cells (d) (C = capsule; magnification ×240). (B) Anti-LERK-2 is also identified on discrete foci of mesenchymal cell surfaces (arrowheads), as well as on microvascular endothelial cells (arrows) and within capillary loop stage glomeruli (G) (×600). (C) Anti-flk-1 also binds brightly to focal domains on mesenchymal cells (arrowheads), to microvessel endothelia (arrows), and to forming glomeruli (G) (C = capsule. ×240; inset ×600).

vulgaris lectin (WGA; Sigma, St. Louis, MO, USA) for two hours at 4°C. Lectin-agarose beads were washed three times with buffer WG, omitting bovine albumin in the last wash, then bound glycoproteins were eluted in albumin-free WG buffer containing 3 mM N, N′, N″ triacetylchitotriose [25]. Specifically eluted glycoproteins were analyzed by phosphotyrosine Western blot. Equivalent protein loads were resolved by SDS-PAGE, transferred to Immobilon-P transfer membranes (Millipore), and blocked in 3%

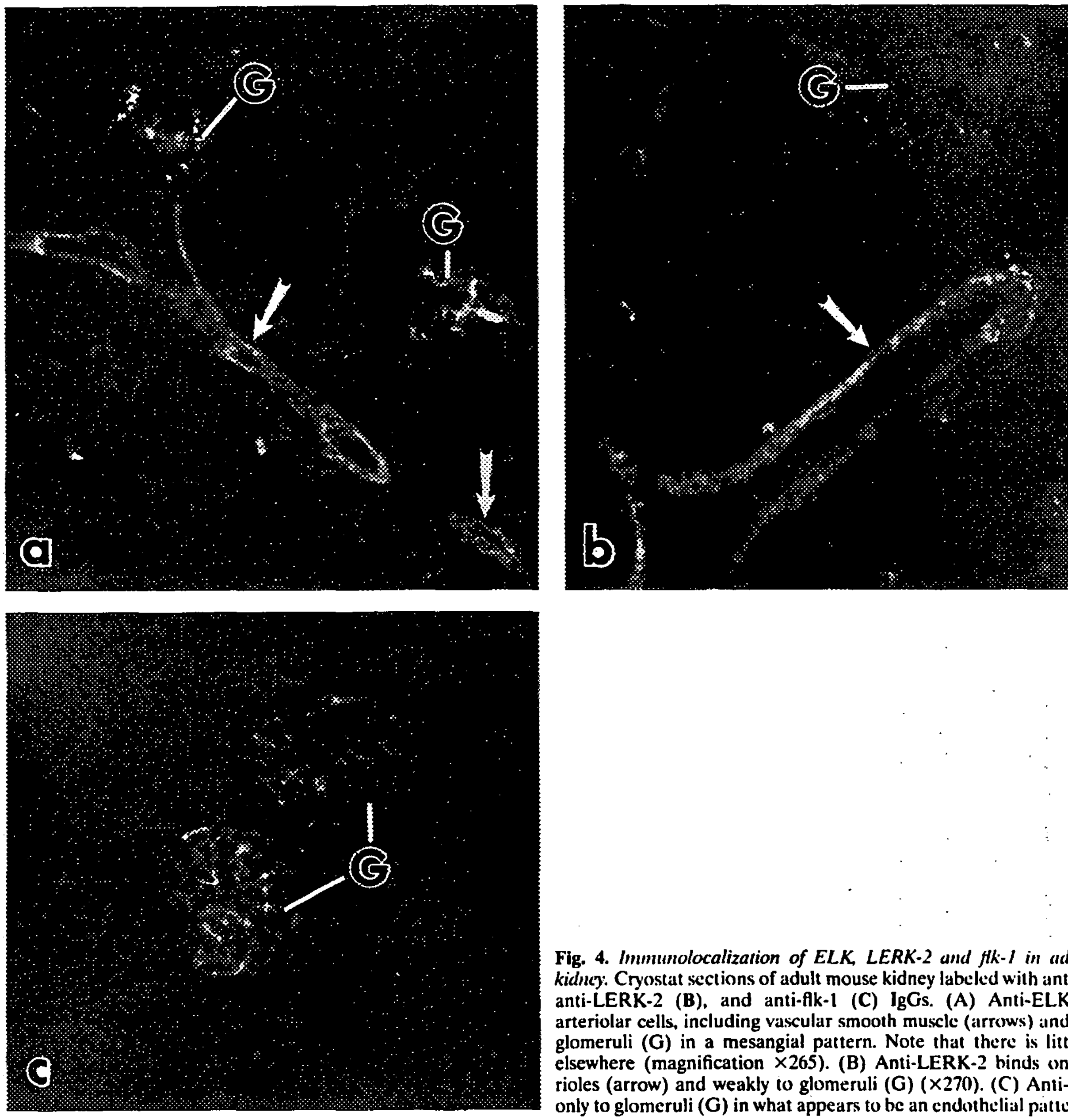


Fig. 4. *Immunolocalization of ELK, LERK-2 and flk-1 in adult murine kidney.* Cryostat sections of adult mouse kidney labeled with anti-ELK (A), anti-LERK-2 (B), and anti-flk-1 (C) IgGs. (A) Anti-ELK binds to arteriolar cells, including vascular smooth muscle (arrows) and to mature glomeruli (G) in a mesangial pattern. Note that there is little labeling elsewhere (magnification ×265). (B) Anti-LERK-2 binds only to arterioles (arrow) and weakly to glomeruli (G) (×270). (C) Anti-flk-1 binds only to glomeruli (G) in what appears to be an endothelial pattern (×270).

BSA in Tris-buffered saline, (20 mM Tris, pH 7.6/137 mM NaCl/ 0.1% Tween-20) for one hour at room temperature. Blots were incubated with the phosphotyrosine monoclonal antibody 4G10 (UBI), followed by incubation with horseradish peroxidase-conjugated mouse anti-rabbit or goat anti-mouse IgG antibodies (Boehringer). Membranes were washed, then developed using a chemiluminescent reaction (ECL, Amersham Corp.) according to the manufacturer's protocol.

Results

To evaluate whether the expression of HRMEC ELK receptors was complemented by expression of the ELK ligand, LERK-2, we analyzed total RNA recovered from these cells cultured under several different conditions. Shown in Figure 1 are Northern blots demonstrating high level expression of both ELK and LERK-2 mRNAs in cells plated for eight hours on Matrigel-coated plastic dishes. Similar receptor and ligand levels were identified in cells plated on gelatin-coated surfaces (data not shown). Hence, both ligand and receptor are expressed by HRMEC under these culture conditions, indicating the potential for autocrine or juxtacrine action of these cell surface proteins, and raising the expectation that renal microvascular cells may express both ligand and receptor *in situ*.

Also in Figure 1, we asked whether the levels of either ELK or LERK-2 mRNA were altered by the protein kinase C activator, phorbol myristate acetate (PMA), an agonist that stimulates angiogenesis *in vivo* [26] and assembly of endothelial cells into a capillary-like networks *in vitro* [27]. The capacity of the LERK family of ligands to act through an autocrine mechanism was recently demonstrated as TNFα induces endothelial expression of

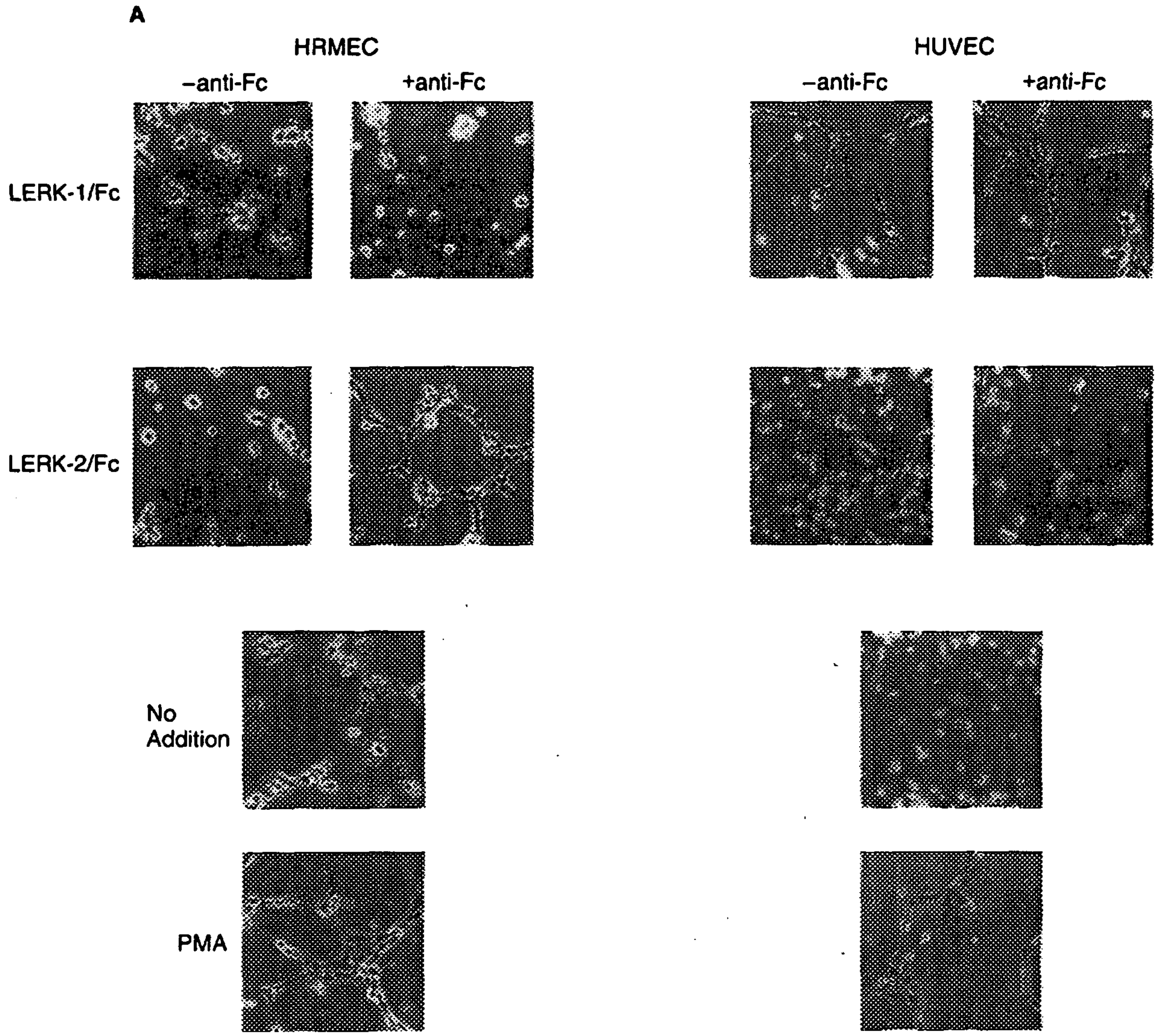


Fig. 5. *HRMEC and HUVEC show distinct responses to LERK-2 and LERK-1.* **A.** Endothelial cells of each type were plated under identical conditions as indicated in the **Methods** section, in basal medium supplemented with 250 ng ml^{-1} of either LERK-2/Fc or LERK-1/Fc without (−) or with (+) prior clustering with 25 ng ml^{-1} of anti-human IgG_1 that recognizes the Fc domain. Control cultures (lower panels) were supplemented with either no addition (as indicated) or 20 ng ml^{-1} phorbol myristate acetate (PMA), as indicated. Phase contrast photomicrographs (×200-fold magnification) were obtained eight hours after plating using TMX-100 (Kodak) film. **B.** Endothelial cells of each type were plated on gelatin coated dishes in basal media supplemented with the indicated concentrations of either LERK-1 (B61)/Fc, or LERK-2/Fc, again either preclustered with anti-human IgG_1 (+anti-Fc) or not (−anti-Fc) at a concentration 10-fold less than that of the respective Fc fusion protein (**Methods**). Cells were lysed and WGA lectin fractions recovering Eck (stimulated by LERK-1 (B61)/Fc) or ELK (stimulated by LERK-2/Fc) were analyzed by phosphotyrosine Western blot, using the 4G10 antibody.

the B61 (LERK-1) ligand for a different Eph family receptor, Eck, and mediates TNFα angiogenic responses [21]. Yet unlike the TNFα effect on LERK-1, PMA did not alter the constitutively high level of LERK-2 (or ELK) expression in the HRMEC cultures (Fig. 1), during the eight hour period required for assembly of capillary-like structures. This finding suggests that PMA-induced assembly is not mediated by increased LERK-2 expression.

To permit the localization of ELK and LERK-2 expression in relevant structures during *in vivo* renal microvascular development, we evaluated the utility of rabbit polyclonal antisera previously raised against a rat ELK fusion protein [23, 28]. In independent experiments, we recently cloned the human ELK receptor from a HRMEC library, and generated an expression construct driving transient expression of an epitope tagged (hemagglutinin, HA) HuELK (Stein, et al, manuscript submitted). It is noteworthy that human ELK receptor amino acid sequence is 97% identical to rat ELK [29]. In Figure 2, the rabbit anti-rat ELK antibodies immunoprecipitated the transiently expressed, HA-tagged human ELK protein from transfected COS 7 cells; the recovered ELK receptors were detected using an anti-HA monoclonal antibody. These ELK antibodies do not identify ELK receptors on Western blots, but were useful for immunolocalization experiments, as shown below.

The rabbit polyclonal anti-LERK-2 antibodies used in these experiments are directed against an 18 amino acid peptide

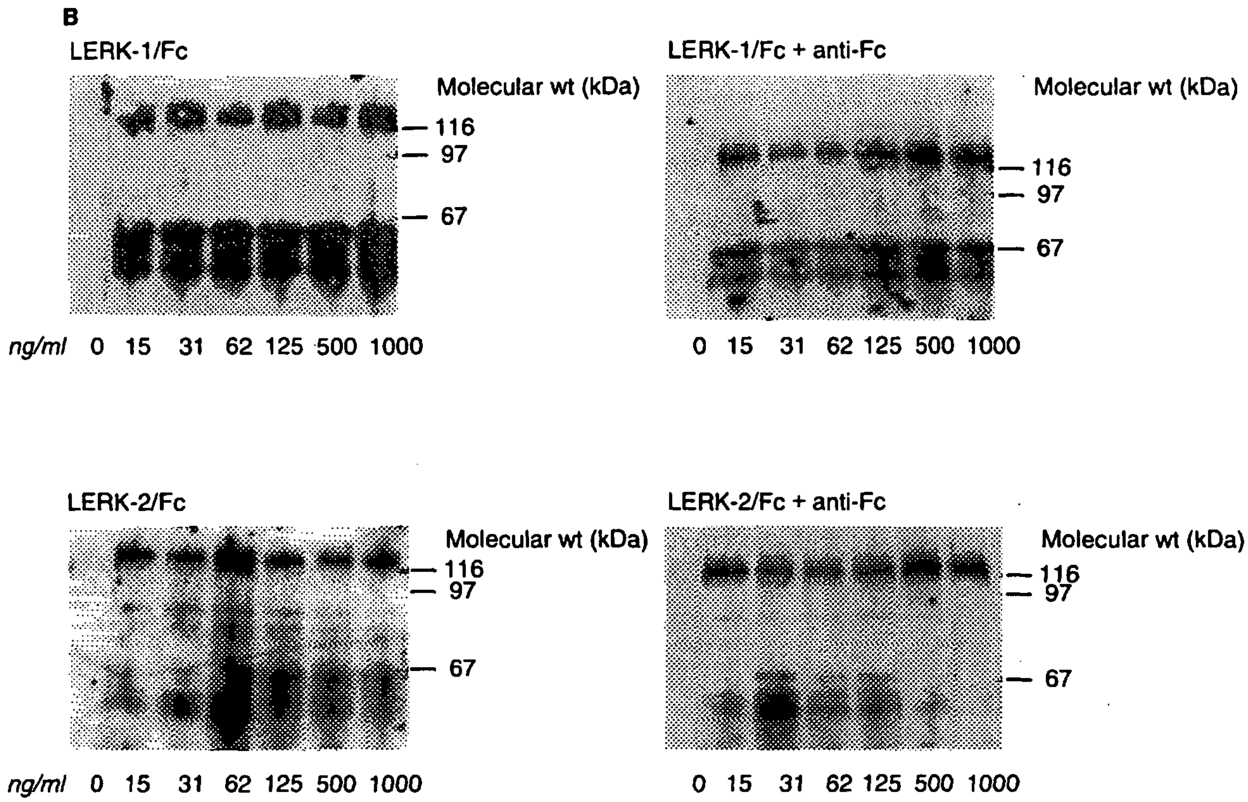


Fig. 5. *Continued*

sequence from the extracellular juxtamembrane spacer domain [28]; that domain is unique to human LERK-2, among the seven previously cloned LERK family proteins (Cerretti, unpublished results). These LERK-2 antibodies cross react with murine LERK-2 and were previously characterized. The polyclonal antibodies to ELK and LERK-2 both recognize cell surface accessible epitopes in the cultured HRMEC, based on their binding to nonpermeabilized cells (data not shown).

Immunolocalization of ELK and LERK-2

To evaluate the relevance of ELK expression and LERK-2 responses in the HRMEC cultures, we asked if this ligand receptor pair is expressed during development of renal microcirculatory structures *in vivo*. We took advantage of the postnatal superficial cortical renal glomerular development seen in murine kidney, the high degree of interspecies sequence identity and interspecies antibody cross reactivity.

Immunofluorescence microscopy was conducted on frozen sections taken from both newborn and adult mouse kidney.

On sections of newborn kidney, anti-ELK and anti-LERK-2 both bound intensely to focal spots on mesenchymal cells in the subcapsular cortex (Fig. 3 A, B). Anti-ELK faintly labeled developing glomeruli, and the apical surfaces of collecting duct epithelial cells were also positive (Fig. 3A). In addition, LERK-2 bound to microvascular endothelial cells and to the developing capillary walls of forming glomeruli (Fig. 3B). These binding patterns were extremely similar to those we have observed previously for the VEGF receptor, flk-1 (Robert et al, manuscript submitted for publication). As seen in Figure 3C, flk-1 also immunolocalized brilliantly to discrete foci on mesenchymal cells, forming microvessels, and developing glomeruli. When the distribution of flk-1 (shown at higher magnification in the inset on Fig. 3C) was compared with that for LERK-2 (Fig. 3B), the patterns appear essentially identical.

When adult mouse kidneys were examined, the labeling was again very similar for both ELK and LERK-2 although there were some substantial changes from what was seen in newborns. Anti-ELK and anti-LERK-2 appeared to label arteriolar smooth muscle cells (Fig. 4 A, B) and glomerular cells distributed in a mesangial pattern were also positive for ELK (Fig. 4A). In contrast, LERK-2 was also found in glomeruli, but the pattern was weaker and more diffuse (Fig. 4B). Anti-flk-1 was only found in glomeruli of adult kidneys, where it appeared to distribute to endothelial cells (Fig. 4C). Anti-flk-1 did not label other mature vascular structures. In striking contrast to what was seen in newborn kidneys, neither ELK, LERK-2, nor flk-1 was localized to discrete foci on dispersed mesenchymal or interstitial cells in mature kidney (Fig. 4 A–C).

Capillary-like assembly responses to LERK-2/Fc and LERK-1/Fc distinguish HRMEC from HUVEC

In an effort to determine whether renal microvascular endothelial responses may differentiate the ELK ligand, LERK-2, from LERK-1 (B61), we evaluated effects of Fc fusion forms of each on the assembly of capillary-like structures on Matrigel-coated plastic surfaces. Given previous evidence that LERK-1 is induced by angiogenic stimuli in HUVEC [21], and that endothelial cells

from distinct vascular sources display remarkably heterogeneous phenotypes [30–33], we compared effects of both ligands on HUVEC in parallel experiments.

Shown in Figure 5 are representative fields eight hours after plating each endothelial type on Matrigel, in medium supplemented with biologically active fusion forms of either LERK-2 (LERK-2/Fc) or LERK-1 (LERK-1/Fc) [23, 28]. We recently showed that multimerization of ligand with a clustering anti-Fc antibody is required to evoke cellular assembly responses to LERK-2/Fc, despite similar activation of ELK receptor tyrosine kinase by clustered and unclustered LERK-2/Fc (Stein et al, manuscript submitted for publication). An irrelevant open reading frame Fc fusion protein has no effect on either endothelial cell type in the absence or presence of clustering anti-Fc (data not shown).

LERK-2/Fc, stimulated assembly of HRMEC into capillary-like structures when clustered by an anti-Fc antibody (ED_{50} = 125 ng/ml), but failed to support similar assembly of HUVEC under identical conditions (Fig. 5A). In contrast, LERK-1/Fc promoted assembly of HUVEC, but not HRMEC, into capillary-like structures. Therefore, responses were determined independently; both the ligand (LERK-1 or LERK-2) and the vascular site of origin of the cultured endothelial cells (HRMEC vs. HUVEC) were critical determinants of the endothelial assembly response.

The obvious issue suggested by these endothelial differences in ligand responsiveness was whether or not receptors for each ligand were expressed at similar levels in the two cell types, and if so, whether they were similarly responsive to ligand-induced activation and tyrosine phosphorylation. To further explore this question, we evaluated the effects of each ligand upon ligand-induced ELK or Eck receptor tyrosine phosphorylation. For this experiment, we took advantage of the convenient property of each receptor to bind WGA lectin (triticum vulgaris lectin) with high affinity.

Shown in Figure 5B are dose responses evaluating tyrosine phosphorylation of WGA lectin-recovered Eph family receptors, ELK and Eck, from HRMEC following exposure to their respective ligands, LERK-2/Fc and LERK-1/Fc. Both ELK and Eck receptors migrate at approximately 130 kDa, and were similarly sensitive to low concentrations of both LERK-2/Fc (lower panels) and LERK-1/Fc (upper panels) (15 ng ml^{-1}), whether or not these ligands were clustered into multimeric complexes using the anti-Fc antibodies (+ anti-Fc, as indicated). Identical experiments conducted on HUVEC under identical conditions showed that both LERK-2/Fc and LERK-1/Fc responsive receptors (ELK and Eck) were similarly tyrosine phosphorylated, independent of the presence or absence of clustering antibody (anti–Fc) (data not shown). From these results, and indendent Northern analysis data in each cell type, we conclude that both ELK and Eck are similarly expressed and responsive in both endothelial cell types. The variable assembly responses are most likely to reflect differential coupling of these receptor activation events through signaling systems or response systems that are endothelial cell type distinct.

Discussion

These experiments provide support for the participation of ELK receptors in assembly of endothelial cells and endothelial progenitor cells into microvascular structures during kidney development. Several lines of evidence support this possibility. Primary cultures of microvascular endothelial cells from human kidney express the ELK ligand, LERK-2, as well as ELK receptors (Fig. 1). However, simple expression of LERK-2 appears insufficient for endogenous ELK receptors to be activated and mediate assembly responses. We recently determined that endogenous ELK receptors are activated by endogenous ligands in the course of PMA and EGF stimulated *in vitro* assembly of capillary-like structures, showing a requirement for ELK in the process (Stein et al, manuscript submitted for publication). Further definition of the processes responsible for ELK's activation and participation in endothelial structure assembly is in progress.

Distinct responses to different ligands for Eph family kinases were displayed by renal microvascular endothelial cells, compared with umbilical vein endothelial cells (Fig. 5). Given that both HUVEC and HRMEC express both ELK and Eck receptors, and that both receptors are tyrosine phosphorylated to similar extents by their respective ligands under these experimental conditions (data not shown), these findings suggest that endothelial cells differentiated in different vascular beds may discriminate assembly responses based on the signaling systems coupled to activation of specific receptors within the Eph family.

If this hypothesis is correct, appropriate Eph family receptors and their ligands should be expressed in cells that will contribute to the developmental assembly of microvascular structures *in vivo*. Our data show that the distribution of expression of ELK and LERK-2 in developing newborn murine kidney is very similar to that of flk-1, the earliest available marker for differentiating endothelial progenitor cells. Whether the same cells are positive for all three proteins remains to be determined. Similarly, the precise cellular location of flk-1, ELK and LERK-2 at different stages of microvascular assembly requires electron microscopic level definition that is in progress. Nevertheless, the finding that ELK, LERK-2, and flk-1 all appear to be concentrated or clustered in discrete sites on mesenchymal cells suggests that these proteins colocalize to points of intercellular contact, positions at which critical signals for multicellular assembly are required.

Although the overall distribution of cells labeling for ELK, LERK-2, and flk-1 in newborn kidney is strikingly similar, there are notable differences in intensities of labeling. This is particularly true for the maturing stage glomeruli of newborn kidney which label strongly for flk-1 (Fig. 4C), but weakly for ELK (Fig. 4A). As maturing stage glomeruli continue to develop, however, the expression of flk-1 diminishes, and only relatively low levels of flk-1 are seen in adult glomeruli (Fig. 5C). In contrast, the expression of ELK seems to increase into adulthood, where high levels of ELK are seen in fully mature glomeruli (Fig. 5A). Although there is a corresponding high level of LERK-2 expression in arterioles of adult kidney, there is markedly less LERK-2 in glomeruli than ELK. The reasons for the temporally variable levels of expression of these receptors and ligands are not currently known, but one may speculate that some of the early proliferative and migratory events in vascular assembly are attributable to flk-1/VEGF signaling, whereas somewhat later in sequence, the ELK/LERK-2 interactions promote cell-cell recognition and establishment of stable interendothelial contacts and connections. The explanation for continued glomerular expression of flk-1 and ELK/LERK-2 is also uncertain. Previous data demonstrated VEGF expression in adult glomeruli [34]. Hence, VEGF/flk-1 and LERK-2/ELK ligand-receptor pairs may be

important in maintaining the differentiated endothelial cell phenotype.

Because ELK and LERK-2 in developing newborn kidneys appear to colocalize with flk-1, and based on the *in vitro* responses mediated through this ligand/receptor pair, we suggest that the coordinated and overlapping expression of these receptors/ligands are likely to be important temporal and spatial controls for the morphogenesis of the renal microvasculature. Moreover, the evidence presented here supports the possibility that distinct endothelial lineages are programmed to distinguish among different ligands for Eph family receptors, potentially providing a molecular basis for the coordinated developmental assembly of microvascular endothelial structures through a vasculogenic process.

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